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(54) Title: METHODS AND COMPOSITIONS FOR THE TREATMENT OF PANCREATITIS		
(57) Abstract Methods and compositions for the treatment of acute pancreatitis in a mammal. Particular compositions comprise a binding element, a translocation element, and a therapeutic element able to prevent accumulation of digestive enzymes within the pancreas.		

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METHODS AND COMPOSITIONS
FOR THE TREATMENT OF PANCREATITIS

This application is a continuation-in-part of
10 application serial no. 09/288,326, filed April 8, 1999.

Field of the Invention

The present invention includes methods and
15 compositions for the treatment of acute pancreatitis.
In a preferred embodiment the invention concerns the use
of agents to reduce or prevent the secretion of
pancreatic digestive enzymes within the pancreas. Such
agents are targeted to pancreatic cells, and serve to
20 prevent the exocytotic fusion of vesicles containing
these enzymes with the plasma membrane. The invention
is also concerned with methods of treating a mammal
suffering from pancreatitis through the administration
of such agents.

25

Background of the Invention

Pancreatitis is a serious medical condition
involving an inflammation of the pancreas. In acute or
30 chronic pancreatitis the inflammation manifests itself
in the release and activation of pancreatic enzymes
within the organ itself, leading to autodigestion. In
many cases of acute pancreatitis, the condition can lead
to death.

35 In normal mammals, the pancreas, a large gland
similar in structure to the salivary gland, is
responsible for the production and secretion of

5 digestive enzymes, which digest ingested food, and bicarbonate for the neutralization of the acidic chyme produced in the stomach. The pancreas contains acinar cells, responsible for enzyme production, and ductal cells, which secrete large amounts of sodium bicarbonate
10 solution. The combined secretion product is termed "pancreatic juice"; this liquid flows through the pancreatic duct past the sphincter of Oddi into the duodenum. The secretion of pancreatic juice is stimulated by the presence of chyme in the upper
15 portions of the small intestine, and the precise composition of pancreatic juice appears to be influenced by the types of compounds (carbohydrate, lipid, protein, and/or nucleic acid) in the chyme.

The constituents of pancreatic juice includes
20 proteases (trypsin, chymotrypsin, carboxypolypeptidase), nucleases (RNase and DNase), pancreatic amylase, and lipases (pancreatic lipase, cholesterol esterase and phospholipase). Many of these enzymes, including the proteases, are initially synthesized by the acinar cells
25 in an inactive form as zymogens: thus trypsin is synthesized as trypsinogen, chymotrypsin as chymotrypsinogen, and carboxypolypeptidase as procarboxypolypeptidase. These enzymes are activated according to a cascade, wherein, in the first step,
30 trypsin is activated through proteolytic cleavage by the enzyme enterokinase. Trypsinogen can also be autoactivated by trypsin; thus one activation has begun, the activation process can proceed rapidly. Trypsin, in turn, activates both chymotrypsinogen and
35 procarboxypolypeptidase to form their active protease counterparts.

5 The enzymes are normally activated only when they enter the intestinal mucosa in order to prevent autodigestion of the pancreas. In order to prevent premature activation, the acinar cells also co-secrete a trypsin inhibitor that normally prevents activation of
10 the proteolytic enzymes within the secretory cells and in the ducts of the pancreas. Inhibition of trypsin activity also prevents activation of the other proteases.

 Pancreatitis can occur when an excess amount of
15 trypsin saturates the supply of trypsin inhibitor. This, in turn, can be caused by underproduction of trypsin inhibitor, or the overabundance of trypsin within the cells or ducts of the pancreas. In the latter case, pancreatic trauma or blockage of a duct can
20 lead to localized overabundance of trypsin; under acute conditions large amounts of pancreatic zymogen secretion can pool in the damaged areas of the pancreas. If even a small amount of free trypsin is available activation
25 of all the zymogenic proteases rapidly occurs, and can lead to digestion of the pancreas (acute pancreatitis) and in particularly severe cases to the patient's death.

 Pancreatic secretion is normally regulated by both hormonal and nervous mechanisms. When the gastric phase of stomach secretion occurs, parasympathetic nerve
30 impulses are relayed to the pancreas, which initially results in acetylcholine release, followed by secretion of enzymes into the pancreatic acini for temporary storage.

 When acid chyme thereafter enters the small
35 intestine, the mucosal cells of the upper intestine release a hormone called secretin. In humans, secretin

5 is a 27 amino acid (3400 Dalton) polypeptide initially produced as the inactive form prosecretin, which is then activated by proteolytic cleavage. Secretin is then absorbed into the blood. Secretin causes the pancreas to secrete large quantities of a fluid containing
10 bicarbonate ion. Secretin does not stimulate the acinar cells, which produce the digestive enzymes. The bicarbonate fluid serves to neutralize the chyme and to provide a slightly alkaline optimal environment for the enzymes.

15 Another peptide hormone, cholecystokinin (CCK) is released by the mucosal cells in response to the presence of food in the upper intestine. As described in further detail below, human CCK is synthesized as a protoprotein of 115 amino acids. Active CCK forms are
20 quickly taken into the blood through the digestive tract, and normally stimulate the secretion of enzymes by the acinar cells. However, stimulation of the CCK receptor by the CCK analogs cerulein and CCK-octapeptide (CCK-8) appears to lead to a worsening of morbidity and
25 mortality in mammals in whom pancreatitis is induced. See Tani et al., *Pancreas* 5:284-290 (1990).

As indicated above, the digestive enzymes are synthesized as zymogens; proto-enzyme synthesis occurs in the rough endoplasmic reticulum of the acinar cells.
30 The zymogens are then packaged within vesicles having a single lipid bilayer membrane. The zymogens are packed within the vesicles so densely that they appear as quasi-crystalline structures when observed under light microscopy and the zymogen granules are electron-dense
35 when observed under the electron microscope. The vesicles are localized within the cytoplasm of the

5 acinar cells. Secretion of zymogens by the acinar cells occurs through vesicle docking and subsequent fusion with the plasma membrane, resulting in the liberation of the contents into the extracellular milieu.

Nerve cells appear to secrete
10 neurotransmitters and other intercellular signaling factors through a mechanism of membrane fusion that is shared with other cell types, see e.g., Rizo & Sudhof, *Nature Struct. Biol.* 5:839-842 (October 1998), hereby incorporated by reference herein, including the
15 pancreatic acinar cells.

Although the Applicants do not wish to be bound by theory, it is believed that a vesicle first contacts the intracellular surface of the cellular membrane in a reaction called docking. Following the docking step the
20 membrane fuses with and becomes part of the plasma membrane through a series of steps that currently remain relatively uncharacterized, but which clearly involve certain vesicle and membrane-associated proteins, as has been illustrated using neural models.

25 In neurons, neurotransmitters are packaged within synaptic vesicles, formed within the cytoplasm, then transported to the inner plasma membrane where the vesicles dock and fuse with the plasma membrane. Recent studies of nerve cells employing clostridial neurotoxins
30 as probes of membrane fusion have revealed that fusion of synaptic vesicles with the cell membrane in nerve cells depends upon the presence of specific proteins that are associated with either the vesicle or the target membrane. See *id.* These proteins have been
35 termed SNAREs. As discussed in further detail below, a protein alternatively termed synaptobrevin or VAMP

5 (vesicle-associated membrane protein) is a vesicle-associated SNARE (v-SNARE). There are at least two isoforms of synaptobrevin; these two isoforms are differentially expressed in the mammalian central nervous system, and are selectively associated with
10 synaptic vesicles in neurons and secretory organelles in neuroendocrine cells. The target membrane-associated SNAREs (t-SNAREs) include syntaxin and SNAP-25. Following docking, the VAMP protein forms a core complex with syntaxin and SNAP-25; the formation of the core
15 complex appears to be an essential step to membrane fusion. See Rizo & Sudhof, *id.* and Neimmann et al., *Trends in Cell Biol.* 4:179-185 (May 1994), hereby incorporated by referenced herein.

Recently evidence has increasingly indicated
20 that the SNARE system first identified in neural cells is a general model for membrane fusion in eukaryotic cells. A yeast exocytotic core complex similar to that of the synaptic vesicles of mammalian neural cells has been characterized, and found to contain three proteins:
25 Sso 1 (syntaxin 1 homolog), SncI (synaptobrevin homolog), and sec9 (SNAP-25 homolog). Rizo & Sudhof, *id.*

These proteins share a high degree of amino acid sequence homology with their mammalian synaptosomal counterparts.

30 All mammalian non-neuronal cells appear to contain cellubrevin, a synaptobrevin analog - this protein is involved in the intracellular transport of vesicles, and is cleaved by TeTx, BoNT/E, BoNT/F, and BoNT/G. Homologs of syntaxin have been identified in
35 yeast (e.g., sso1p and sso2p) and mammalian non-neuronal cells (syn2p, syn3p, syn4p and syn5p). Finally, as

5 indicated above, a yeast SNAP-25 homolog, sec9 has been identified; this protein appears to essential for vesicle fusion with the plasma membrane.

Intoxication of neural cells by clostridial neurotoxins exploits specific characteristics of the
10 SNARE proteins. These neurotoxins, most commonly found expressed in *Clostridium botulinum* and *Clostridium tetanus*, are highly potent and specific poisons of neural cells. These Gram positive bacteria secrete two related but distinct toxins, each comprising two
15 disulfide-linked amino acid chains: a light chain (L) of about 50 KDa and a heavy chain (H) of about 100 KDa, which are wholly responsible for the symptoms of botulism and tetanus, respectively.

The tetanus and botulinum toxins are among the most
20 lethal substances known to man; both toxins function by inhibiting neurotransmitter release in affected neurons.

The tetanus neurotoxin (TeNT) acts mainly in the central nervous system, while botulinum neurotoxin (BoNT) acts at the neuromuscular junction; both toxins
25 inhibit acetylcholine release from the nerve terminal of the affected neuron into the synapse, resulting in paralysis or reduced target organ function.

The tetanus neurotoxin (TeNT) is known to exist in one immunologically distinct type; the botulinum
30 neurotoxins (BoNT) are known to occur in seven different immunologically distinct serotypes, termed BoNT/A through BoNT/G. While all of these latter types are produced by isolates of *C. botulinum*, two other species, *C. baratii* and *C. butyricum* also produce toxins similar
35 to /F and /E, respectively. See e.g., Coffield et al.,
The Site and Mechanism of Action of Botulinum

5 *Neurotoxin in Therapy with Botulinum Toxin 3-13*

(Jankovic J. & Hallett M. eds. 1994), the disclosure of which is incorporated herein by reference.

 Regardless of type, the molecular mechanism of intoxication appears to be similar. In the first step
10 of the process, the toxin binds to the presynaptic membrane of the target neuron through a specific interaction between the heavy chain and a neuronal cell surface receptor; the receptor is thought to be different for each type of botulinum toxin and for TeNT.
15 The carboxy terminal (C-terminal) half of the heavy chain is required for targeting of the toxin to the cell surface. The cell surface receptors, while not yet conclusively identified, appear to be distinct for each neurotoxin serotype.

20 In the second step, the toxin crosses the plasma membrane of the poisoned cell. The toxin is first engulfed by the cell through receptor-mediated endocytosis, and an endosome containing the toxin is formed. The toxin (or light chain thereof) then escapes
25 the endosome into the cytoplasm of the cell. This last step is thought to be mediated by the amino terminal (N-terminal) half of the heavy chain, which triggers a conformational change of the toxin in response to a pH of about 5.5 or lower. Endosomes are known to possess a
30 proton pump that decreases intra-endosomal pH. The conformational shift exposes hydrophobic residues in the toxin, which permits the toxin to embed itself in the endosomal membrane. The toxin then translocates through the endosomal membrane into the cytosol.

35 Either during or after translocation the disulfide bond joining the heavy and light chain is reduced, and

5 the light chain is released into the cytoplasm. The entire toxic activity of botulinum and tetanus toxins is contained in the light chain of the holotoxin; the light chain is a zinc (Zn++) endopeptidase which selectively cleaves the SNARE proteins essential for recognition and
10 docking of neurotransmitter-containing vesicles with the cytoplasmic surface of the plasma membrane, and fusion of the vesicles with the plasma membrane. The light chain of TxNT, BoNT/B, BoNT/D, BoNT/F, and BoNT/G cause specific proteolysis of VAMP, an integral protein.
15 During proteolysis, most of the VAMP present at the cytosolic surface of the synaptic vesicle is inactivated as a result of any one of these cleavage events. Each toxin cleaves a different specific peptide bond.

BoNT/A and /E selectively cleave the plasma
20 membrane-associated SNARE protein SNAP-25; this protein is bound to and present on the cytoplasmic surface of the plasma membrane. BoNT/C1 cleaves syntaxin, which exists as an integral protein having most of its mass exposed to the cytosol. Syntaxin interacts with the
25 calcium channels at presynaptic terminal active zones. See Tonello et al., *Tetanus and Botulism Neurotoxins in Intracellular Protein Catabolism* 251-260 (Suzuki K & Bond J. eds. 1996), the disclosure of which is incorporated by reference as part of this specification.
30 Bo/NTC1 also appears to cleave SNAP-25.

Both TeNT and BoNT are specifically taken up by cells present at the neuromuscular junction. BoNT remains within peripheral neurons and, as indicated above, blocks release of the neurotransmitter
35 acetylcholine from these cells.

By contrast TeNT, through its receptor, enters

5 vesicles that move in a retrograde manner along the axon to the soma, and is discharged into the intersynaptic space between motor neurons and the inhibitory neurons of the spinal cord. At this point, TeNT binds receptors of the inhibitory neurons, is again internalized, and
10 the light chain enters the cytosol to block the release of the inhibitory neurotransmitters 4-aminobutyric acid (GABA) and glycine from these cells. Id.

International Patent Publication No. WO 96/33273 relates to derivatives of botulinum toxin designed to
15 prevent neurotransmitter release from sensory afferent neurons to treat chronic pain. Such derivatives are targeted to nociceptive neurons using a targeting moiety that binds to a binding site of the surface of the neuron.

20 International Patent Publication No. 98/07864 discusses the production of recombinant toxin fragments that have domains that enable the polypeptide to translocate into a target cell or which increase the solubility of the polypeptide, or both.

25

Summary of the Invention

The present invention concerns methods and
30 compositions useful for the treatment of acute pancreatitis. This condition is largely due to the defective secretion of zymogen granules by acinar cells, and by the premature co-mingling of the secreted zymogens with lysosomal hydrolysates capable of
35 activating trypsin, thereby triggering the protease activation cascade and resulting in the destruction of

5 pancreatic tissue.

In one embodiment of this aspect, the invention is a therapeutic agent comprising a chimeric protein containing an amino acid sequence-specific endopeptidase activity which will specifically cleave at least one
10 synaptic vesicle-associated protein selected from the group consisting of SNAP-25, syntaxin or VAMP, in combination with the translocation activity of the N-terminus of a clostridial neurotoxin heavy chain, wherein the chimeric protein further comprises a
15 recognition domain which will bind a human cholecystokinin (CCK) receptor. Upon binding of the recognition domain of the protein to the CCK receptor, the protein is specifically transported into cells containing CCK receptors (pancreatic acinar cells)
20 through receptor-mediated endocytosis. In a preferred embodiment, the CCK receptor is the CCK A receptor.

Once inside the acinar cell, the chimeric protein functions in a manner similar to that of a clostridial neurotoxin within its target neuron. The toxin moiety
25 is translocated from the endosome into the cytoplasm, where it acts to cleave a SNARE protein identical or homologous to SNAP-25, syntaxin or VAMP. The cleavage of this protein prevents formation of a core complex between the SNARE proteins and thus prevents or reduces
30 the extent of fusion of the vesicle with the target membrane. This, in turn, results in inhibition of zymogen release from the acinar cells and of zymogen activation by lysosomal hydrolases. The autodigestion of pancreatic tissue in acute pancreatitis is therefore
35 reduced or eliminated.

Another embodiment of the present invention

5 concerns a method of treating a patient suffering from acute pancreatitis by administering an effective amount of such a chimeric protein.

Another embodiment of the invention concerns a therapeutic composition that contains the translocation
10 activity of a clostridial neurotoxin heavy chain in combination with a recognition domain able to bind a specific cell type and a therapeutic element having an activity other than the endopeptidase activity of a clostridial neurotoxin light chain. A non-exclusive list
15 of certain such therapeutic elements includes: hormones and hormone-agonists and antagonists, nucleic acids capable of being used as replication, transcription, or translational templates (e.g., for expression of a protein drug having the desired
20 biological activity or for synthesis of a nucleic acid drug as an antisense agent), enzymes, toxins, and the like.

In a preferred embodiment, the specific cell type is a pancreatic cell, most preferably a pancreatic
25 acinar cell.

Another embodiment is drawn to methods for the treatment of acute pancreatitis comprising contacting an acinar cell with an effective amount of a composition comprising a chimeric protein containing an amino acid
30 sequence-specific endopeptidase activity which will specifically cleave at least one synaptic vesicle-associated protein selected from the group consisting of SNAP-25, syntaxin or VAMP, in combination with the translocation activity of the N-terminus of a
35 clostridial neurotoxin heavy chain, wherein the chimeric protein further comprises a recognition domain able to

5 bind to a cell surface protein characteristic of an human pancreatic acinar cell. Preferably the cell surface protein is a CCK receptor protein; most preferably the protein is the human CCK A protein. CCK receptors (CCK-A receptor and CCK-B receptor) are found
10 mainly in on the surface of pancreatic acinar cells, although they are also found in some brain cells and, to a lesser extent on the surface of gastrointestinal cells.

Any suitable route of administration may be used in
15 this aspect of the invention. Applicants currently prefer to administer the therapeutic agent in an intravenous infusion solution; however methods such as ingestion (particularly when associated with neurotoxin-associated proteins (NAPs); see Sharma et al., *J. Nat.*
20 *Toxins* 7:239-253 (1998), incorporated by reference herein), direct delivery to the pancreas, injection and the like may also be used. The agent is substantially specifically targeted to pancreatic cells; when the agent contains a CCK receptor-binding domain, the blood-
25 brain barrier prevents the agent from interacting with brain cells.

In yet another embodiment the invention provides a composition comprising a drug or other therapeutic agent having an activity other than that of a clostridial
30 neurotoxin light chain for intracellular delivery, said agent joined to the translocation domain of a clostridial neurotoxin heavy chain and a binding element able to recognize a cell surface receptor of a target cell. In a preferred embodiment, the target cell is not
35 a neuron. Also, in this embodiment it is preferred that the drug or other therapeutic agent has an enzymatic,

5 catalytic, or other self-perpetuating mode of activity,
so that the effective dose of drug is greater than the
number of drug molecules delivered within the target
cell. A non-exclusive list of certain such drugs would
include: hormones and hormone-agonists and antagonists,
10 nucleic acids capable being of being used as
replication, transcription, or translational templates
(e.g., for expression of a protein drug having the
desired biological activity or for synthesis of a
nucleic acid drug as an antisense agent), enzymes,
15 toxins (such as diphtheria toxin or ricin), and the
like.

In this embodiment the drug may be cleavably linked
to the remainder of the composition in such a way as to
allow for the release of the drug from the composition
20 within the target cell.

The presently claimed compositions may be provided
to the patient by intravenous administration, may be
administered during surgery, or may be provided
parenterally.

25 WO 95/32738, which shares ownership with the
present application, describes transport proteins for
the therapeutic treatment of neural cells. This
application is incorporated by reference herein as part
of this specification.

30

Detailed Description of the Preferred Embodiments

In a basic and presently preferred form, the
invention comprises a therapeutic polypeptide comprising
35 three features: a binding element, a translocation
element, and a therapeutic element.

5 The binding element is able to bind to a specific target cell provided that the target cell is not a motor neuron or a sensory afferent neuron. Preferably, the binding element comprises an amino acid chain; also an independently, it is preferably located at or near the
10 C-terminus of a polypeptide chain. By "binding element" is meant a chemical moiety able to preferentially bind to a cell surface marker characteristic of the target cell under physiological conditions. The cell surface marker may comprise a polypeptide, a polysaccharide, a
15 lipid, a glycoprotein, a lipoprotein, or may have structural characteristics of more than one of these. By "preferentially interact" is meant that the disassociation constant (K_d) of the binding element for the cell surface marker is at least one order of
20 magnitude less than that of the binding element for any other cell surface marker. Preferably, the disassociation constant is at least 2 orders of magnitude less, even more preferably the disassociation constant is at least 3 orders of magnitude less than
25 that of the binding element for any other cell surface marker to which the therapeutic polypeptide is exposed. Preferably, the organism to be treated is a human.

 In one embodiment the cell surface receptor comprises the histamine receptor, and the binding
30 element comprises an variable region of an antibody which will specifically bind the histamine receptor.

 In an especially preferred embodiment, the cell surface marker is a cholecystokinin (CCK) receptor. Cholecystokinin is a bioactive peptide that functions as
35 both a hormone and a neurotransmitter in a wide variety of physiological settings. Thus, CCK is involved in the

5 regulation of gall bladder contraction, satiety, gastric emptying, and gut motility; additionally it is involved in the regulation of pancreatic exocrine secretion.

There are two types of CCK receptors, CCK A and CCK B; the amino acid sequences of these receptors have been
10 determined from cloned cDNA. Despite the fact that both receptors are G protein-coupled receptors and share approximately 50% homology, there are distinct differences between their physiological activity. The CCK A receptor is expressed in smooth muscle cells of
15 the gall bladder, smooth muscle and neurons within the gastrointestinal tract, and has a much greater affinity ($>10^2$ times higher) for CCK than the related peptide hormone gastrin. The CCK B receptor, found in the stomach and throughout the CNS, has roughly equal
20 ability to bind CCK and gastrin.

The varied activities of CCK can be partly attributed to the fact that CCK is synthesized as procholecystokinin, a protoprotein of 115 amino acids, and is then post-translationally cleaved into a number
25 of active fragments all sharing the same C-terminus. The amino acid sequence of human procholecystokinin is shown below; amino acid residues not present in the biologically active cleavage products are in lower case.

All amino acid sequences herein are shown from N-
30 terminus to C-terminus, unless expressly indicated otherwise:

Human procholecystokinin, having the amino acid sequence SEQ ID NO:1:

35

5 mmsgvclcvlmaavlagaaltqpvppadpagsglqraeeaprrqlr VSQRT
 DGESRAHLGA LLARYIQQAR KAPSGRMSIV KNLQNLDPSH
 RISDRDYMGW MDF grrsaeeeyeps

 Biologically active cleavage products of the full
10 length CCK chain include:
 CCK-58, having the amino acid sequence SEQ ID NO:2:

 VSQRT DGESRAHLGA LLARYIQQAR KAPSGRMSIV KNLQNLDPSH
 RISDRDYMGW MDF;

15 CCK-39, having the amino acid sequence SEQ ID NO:
 3:

 YIQQAR KAPSGRMSIV KNLQNLDPSH RISDRDYMGW MDF;

20 CCK-33, having the amino acid sequence SEQ ID NO:
 4:

 KAPSGRMSIV KNLQNLDPSH RISDRDYMGW MDF;

25 CCK-12, having the amino acid sequence SEQ ID NO:
 5:

 ISDRDYMGW MDF;

30 and CCK-8, having the amino acid sequence SEQ ID
 NO: 6:

 RDYMGW MDF.

35 In each case, the biologically active polypeptides
 contain post-translational modifications; in the case of

5 CCK species binding the CCK-A receptor, amidation of the C-terminal phenylalanine, and sulfatation of the tyrosine residue located seven residue from the C-terminus of the biologically active species are required for hoigh affinity binding ton the receptor. In the
10 case of CCK-B, only the C-terminal amidation is necessary; sulfation of the tyrosine appears to make little diffrence in CCK-B binding. These modifications appear to be necessary for full biological activity, although both the unmodified C-terminal pentapeptide and
15 tetrapeptide of CCK retains some biological activity. Kennedy et al., *J. Biol. Chem.* 272: 2920-2926 (1997), hereby incorporated by reference herein.

In a preferred embodiment, the biologically active therapeutic polypeptide of the present invention
20 comprises a CCK binding element containing the post-translational modifications described above. This polypeptide can be produced by synthetic chemistry or, preferably, can be produced by a combination of recombinant and synthetic means using the "expressed
25 protein ligation" (EPL) method. See Cotton & Muir, *Chemistry & Biology* 6:R247 (1999), hereby incorporated by reference herein. In this method the therapeutic polypeptide is expressed without the C-terminal binding element as a fusion protein with an "intein" polypeptide
30 sequence positioned at the C-terminus thereof. The intein comprises a conserved cysteine, serine, or threonine residue at its amino terminus; the carboxyl terminus of the intein contains a functional binding sequence such as chitin binding domain (CBD), poly His
35 (6 or more consecutive histidine residues), or another amino acid sequence capable of affinity binding. The

5 coding sequence of this recombinantly expressed polypeptide is constructed using standard recombinant DNA methods.

Additionally, standard solid phase peptide synthesis methods are employed to construct a synthetic peptide comprising a C-terminal amidated phenylalanine and the desired CCK amino acid sequence. Such methods are described in e.g., Bodansky, M. and Bodansky, A. *The Practice of Peptide Synthesis* (2d ed. Trost B.M., ed. Springer Laboratory 1994), hereby incorporated by reference herein. The synthetic peptide also contains an sulfated tyrosine at the position 7 residues from the carboxyl terminus. This can be done either by incorporation of commercially available Fmoc-Tyr(OSO₃⁻)-OH into the peptide chain at the 7th amino acid position prior to cleavage of the synthetic peptide from the solid support hereby incorporated by reference herein), or by standard peptide synthesis using tyrosine at position 7, followed by a sulfation reaction of the peptide resulting in tyrosine sulfate at the 7 position. See e.g., Koeller, K.M., *J. Am. Chem. Soc.* 122:742-743 (2000). The synthetic peptide is constructed with a cysteine (or serine or threonine) residue at the amino terminus.

It will be understood that one can use either hydroxyl-containing amino acids or cysteine as the amino terminal residue of the intein and the synthetic peptide, and either thiopheol, phenol or another nucleophile capable of creating a reactive ester or thioester linkage in accordance with the expressed protein ligation methods described herein. However,

5 thiol-containing amino acid residues and thiophenol or another sulfur-containing nucleophile are preferred.

Thus, according to one embodiment of the expressed protein ligation method, the fusion protein is immobilized following expression by incubation under
10 selective binding conditions with a surface to which the binding partner of the carboxyl terminal has been joined (e.g., where the binding moiety is CBP, the surface may be a resin to which chitin is conjugated). The immobilized fusion protein is then permitted to react in
15 a transthioesterification reaction with a S- or O-containing reagent (such as thiophenol or phenol) and the synthetic modified peptide described above. In this step, the intein which is joined to the carboxyl terminus of the therapeutic polypeptide is cleaved at
20 the thioester (or ester) linkage, thus liberating the protein from the surface to which it was bound. The intein may be transiently replaced with the thiophenol group, and the resulting thioester is then itself attacked by the cysteine (or serine or threonine)
25 residue of the synthetic peptide; this reaction is then spontaneously followed by a shift of the carbonyl bond from S (or O) to the N terminal nitrogen of the synthetic peptide, to form a peptide bond. The resultant therapeutic polypeptide thus comprises a
30 therapeutic domain, a translocation domain, and a binding domain comprising a CCK sequence modified to contain the naturally occurring post-translational modifications.

As intended herein, the term "extein" refers to a
35 portion of a chimeric polypeptide that borders one or more intein, and is subsequently ligated to either

5 another extein or a synthetic polypeptide in the EPL reaction referred to herein.

As intended herein, the term "intein" refers to a portion of a chimeric polypeptide containing an N-terminal cysteine, serine, or threonine which is excised
10 from said polypeptide during the EPL reaction referred to herein.

Of course, the Applicants contemplate that this method of producing a CCK-containing therapeutic polypeptide is exemplary only, and that variations and
15 modification of the above-described method will be well within the ability and knowledge of those of ordinary skill in the art in light of the present patent application.

While it will be understood that the applicants do
20 not wish to be bound by theory, the following findings may assist an understanding the nature of the interaction between CCK and the CCK receptors, and thus between the CCK receptor binding element of an embodiment of the present invention and its CCK receptor
25 target.

In pancreatic acinar cells the CCK A receptor undergoes internalization to intracellular sites within minutes after agonist exposure. Pohl et al., *J. Biol. Chem.* 272: 18179-18184 (1997), hereby incorporated by
30 reference herein. The CCK B receptor has also shown the same ligand-dependant internalization response in transfected NIH 3T3 cells. In the CCK B receptor, but not the CCK A receptor, the endocytotic feature of the receptor been shown to be profoundly decreased by the
35 deletion of the C terminal 44 amino acids of the

5 receptor chain, corresponding in both receptors to an cytoplasmic portion of the receptor chain.

Recent studies of the interaction between the CCK A receptor and CCK have shown that the primary receptor sequence region containing amino acid residues 38
10 through 42 is involved in the binding of CCK. Residues Trp₃₉ and Gln₄₀ appear to be essential for the binding of a synthetic CCK C-terminal nonapeptide (in which the methionine residues located at residue 3 and 6 from the C-terminus are substituted by norleucine and threonine
15 respectively) to the receptor. Kennedy et al., *supra*. These residues do not appear to be essential for the binding of CCK analogs JMV 180 (corresponding the synthetic C-terminal heptapeptide of CCK in which the phenylalanylamide residue is substituted by a
20 phenylethyl ester and the threonine is substituted with norleucine), and JMV 179 (in which the phenylalanylamide residue and the L-tryptophan residues of the synthetic CCK nonapeptide are substituted by a phenylethyl ester and D-tryptophan, respectively and the threonine is
25 substituted with norleucine). *Id.*

These and similar studies have shed light on the structure of the CCK A receptor active site. Based on receptor binding experiments, a current structural model indicates that CCK residues Trp₃₀ and Met₃₁ (located at
30 positions 4 and 3, respectively, from the C terminus of mature CCK-8) reside in a hydrophobic pocket formed by receptor residues Leu₃₄₈, Pro₃₅₂, Ile₃₅₃ and Ile₃₅₆. CCK residue Asp₃₂ (located at amino acid position 2 measured from the C terminus of CCK-8) seems to be involved in an
35 ionic interaction with receptor residue Lys₁₁₅. CCK Tyr-

5 sulfate₂₇ (the CCK-8 residue 7 amino acids from C terminus) appears involved in an ionic interaction with receptor residue Lys₁₀₅ and a stacking interaction with receptor residue Phe₁₉₈. Ji, et al., 272 *J. Biol. Chem.* 24393-24401 (1997).

10 Such structural models provide detailed guidance to the person of ordinary skill in the art as to the construction of a variety of binding elements able to retain the binding characteristics of biologically active CCK peptides for the CCK-A receptor, for example,
15 as, for example, by site directed mutagenesis of a clostridial neurotoxin heavy chain. Similarly, models deduced using similar methodologies have been proposed for the CCK B receptor, see e.g., Jagerschmidt, A. et al., *Mol. Pharmacol.* 48:783-789 (1995), and can be used
20 as a basis for the construction of binding elements that retain binding characteristics similar to the CCK B receptor.

It will be appreciated that the CCK-B receptor is known to exist on the surface of neurons associated with
25 the central nervous system. In one alternative embodiment of the present invention the therapeutic polypeptide may be directed (for example, by intrathecal application) to these neurons rather than to the pancreas); in such a case, the binding element may
30 comprise a CCK containing the C terminal amidation only.

Such a binding element may be constructed using the expressed protein ligation (EPL) methods described above. Indeed, EPL methods may be used to introduce and desired or required modifications to the therapeutic
35 element, the translocation element, and/or the binding element of the claimed therapeutic polypeptide.

5 Additionally, the binding element may comprise a variable region of an antibody which will bind the CCK-A or CCK-B receptor.

 Nucleic acids encoding polypeptides containing such a binding element may be constructed using molecular
10 biology methods well known in the art; see e.g., Sambrook et al., *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Laboratory Press 2d ed. 1989), and expressed within a suitable host cell. The disclosure of this latter reference is incorporated by reference
15 herein in its entirety.

 The translocation element comprises a portion of a clostridial neurotoxin heavy chain having a translocation activity. By "translocation" is meant the ability to facilitate the transport of a polypeptide
20 through a vesicular membrane, thereby exposing some or all of the polypeptide to the cytoplasm.

 In the various botulinum neurotoxins translocation is thought to involve an allosteric conformational change of the heavy chain caused by a decrease in pH
25 within the endosome.

 This conformational change appears to involve and be mediated by the N terminal half of the heavy chain and to result in the formation of pores in the vesicular membrane; this change permits the movement of the
30 proteolytic light chain from within the endosomal vesicle into the cytoplasm. See e.g., Lacy, et al., *Nature Struct. Biol.* 5:898-902 (October 1998).

 The amino acid sequence of the translocation-mediating portion of the botulinum neurotoxin heavy
35 chain is known to those of skill in the art; additionally, those amino acid residues within this

5 portion that are known to be essential for conferring the translocation activity are also known.

It would therefore be well within the ability of one of ordinary skill in the art, for example, to employ the naturally occurring N-terminal peptide half of the heavy chain of any of the various *Clostridium tetanus* or *Clostridium botulinum* neurotoxin subtypes as a translocation element, or to design an analogous translocation element by aligning the primary sequences of the N-terminal halves of the various heavy chains and selecting a consensus primary translocation sequence based on conserved amino acid, polarity, steric and hydrophobicity characteristics between the sequences. The therapeutic element of the present invention may comprise, without limitation: active or inactive (i.e., modified) hormone receptors (such as androgen, estrogen, retinoid, peroxisome proliferator and ecdysone receptors etc.), and hormone-agonists and antagonists, nucleic acids capable of being used as replication, transcription, or translational templates (e.g., for expression of a protein drug having the desired biological activity or for synthesis of a nucleic acid drug as an antisense agent), enzymes, toxins (including apoptosis-inducing agents), and the like.

30 In a preferred embodiment, the therapeutic element is a polypeptide comprising a clostridial neurotoxin light chain or a portion thereof retaining the SNARE-protein sequence-specific endopeptidase activity of a clostridial neurotoxin light chain. The amino acid sequences of the light chain of botulinum neurotoxin (BoNT) subtypes A-G have been determined, as has the

5 amino acid sequence of the light chain of the tetanus
neurotoxin (TeNT). Each chain contains the Zn⁺⁺-binding
motif **His-Glu-x-x-His** (N terminal direction at the left)
characteristic of Zn⁺⁺-dependent endopeptidases (HELIH
in TeNT, BoNT/A /B and /E; HELNH in BoNT/C; and HELTH in
10 BoNT/D).

Recent studies of the BoNT/A light chain have
revealed certain features important for the activity and
specificity of the toxin towards its target substrate,
SNAP-25. Thus, studies by Zhou et al. *Biochemistry*
15 34:15175-15181 (1995) have indicated that when the light
chain amino acid residue His₂₂₇ is substituted with
tyrosine, the resulting polypeptide is unable to cleave
SNAP-25; Kurazono et al., *J. Biol. Chem.* 14721-14729
(1992) performed studies in the presynaptic cholinergic
20 neurons of the buccal ganglia of *Aplysia californica*
using recombinant BoNT/A light chain that indicated that
the removal of 10 N-terminal or 32 C-terminal residues
did not abolish toxicity, but that removal of 10 N-
terminal or 57 C-terminal residues abolished toxicity in
25 this system. Most recently, the crystal structure of
the entire BoNT/A holotoxin has been solved; the active
site is indicated as involving the participation of
His₂₂₂, Glu₂₂₃, His₂₂₆, Glu₂₆₁ and Tyr₃₆₅. Lacy et al., *supra*.
(These residues correspond to His₂₂₃, Glu₂₂₄, His₂₂₇, Glu₂₆₂
30 and Tyr₃₆₆ of the BoNT/A L chain of Kurazono et al.,
supra.) Interestingly, an alignment of BoNT/A through E
and TeNT light chains reveals that every such chain
invariably has these residues in positions analogous to
BoNT/A. Kurazono et al., *supra*.

5 The catalytic domain of BoNT/A is very specific for the C-terminus of SNAP-25 and appears to require a minimum of 16 SNAP-25 amino acids for cleavage to occur.

 The catalytic site resembles a pocket; when the light chained is linked to the heavy chain via the disulfide
10 bond between Cys₄₂₉ and Cys₄₅₃, the translocation domain of the heavy chain appears to block access to the catalytic pocket until the light chain gains entry to the cytosol. When the disulfide bond is reduced, the two polypeptide chains dissociate, and the catalytic
15 pocket is then "opened" and the light chain is fully active.

 As described above, VAMP and syntaxin are cleaved by BoNT/B, D, F, G and TeNT, and BoNT/C₁, respectively, while SNAP-25 is cleaved by BoNT/A and E.

20 The substrate specificities of the various clostridial neurotoxin light chains other than BoNT/A are known. Therefore, the person of ordinary skill in the art could easily determine the toxin residues essential in these subtypes for cleavage and substrate
25 recognition (for example, by site-directed mutagenesis or deletion of various regions of the toxin molecule followed by testing of proteolytic activity and substrate specificity), and could therefore easily design variants of the native neurotoxin light chain
30 that retain the same or similar activity.

 Additionally, construction of the therapeutic agents set forth in this specification would be easily constructed by the person of skill in the art. It is well known that the clostridial neurotoxins have three
35 functional domains analogous to the three elements of the present invention. For example, and without

5 limitation, the BoNT/A neurotoxin light chain is present
in amino acid residues 1-448 of the BoNT/A prototoxin
(i.e., before nicking of the prototoxin to form the
disulfide-linked dichain holotoxin); this amino acid
sequence is provided below as SEQ ID NO: 7. Active site
10 residues are underlined:

BoNT/A light chain (SEQ ID NO:7)

MPFVVKQFNYKDPVNGVDIAYIKIPNAGQMOPVKAFKIHNKIWV
15 IPERDTFTNPEEGDLNPPPEAKQVPVSYDSTYLSTDNEKDNYLKGVTKLFERIYSTD
LGRMLLTSIVRGIPFWGGSTIDTELKVIDTNCINVIQPDGSYRSEELNLVIIGPSADI
IQFECKSFGHEVLNLTRNGYGSTQYIRFSPDFTFGFEESLEVDTNPLLGAQKFATDPA
VTLAHELIIHAGHRLYGIAINPNRVFKVNTNAYYEMSGLEVSFEELRTFGGHDQAFIDS
LQENEFRLYYYNKFKDIASTLNKAKSIVGTTASLQYMKNVFKEKYLLSEDTSGKFSVD
20 KLKFDKLYKMLTEIYTEDNFVKFFKVLNRKTYLNFDAVFKINIVPKVNYTIYDGFNL
RNTNLAANFNGQNTTEINNMNFTKLKNFTGLFEFYKLLCVRGIITSKTKSLDKGYNK;

The heavy chain N-terminal (H_N) translocation
domain is contained in amino acid residues 449-871 of
25 the BoNT/A amino acid sequence, shown below as SEQ ID
NO: 8; a gated ion channel-forming domain probably
essential for the translocation activity of this peptide
is underlined (see Oblatt-Montal et al., *Protein Sci.*
4:1490-1497(1995), hereby incorporated by reference
30 herein.

ALNDLCIKVNNWDLFFSPSEDNFTNDLNKGEEITSDTNIEAAEENISLDLIQQYYLTFNF
DNEPENISIEENLSSDIIGQLELMPNIEFPNGKKYELDKYTMFHYLRAQEFEGHKSRI
ALTNSVNEALLNPSRVYTFSSDYVKKVNKATEAAMFLGWVEQLVYDFTDETSEVSTT
35 DKIAIDITIIIPYIGPALNIGNMLYKDDFVGALIFSGAVILLEFIPEIAIPVLGTFALV
SYIANKVLTVQTIDNALSQRNEKWDEVYKYIVTNWLAKVNTQIDLIRKKMKEALENQA
EATKAIINYQYNQYTEEEKNINFNIDDLSSKLNESINKAMININKFLNQCSVSYLMN
SMIPYGVKRLDFDASLKDALLKYIYDNRGTLIGQVDRDKDKVNNTLSTDIPFQLSKY
VDNQRLSTFTTEYIK;

40

5 The heavy chain C-terminal neural cell binding
domain is contained in amino acid residues 872-1296 (SEQ
ID NO: 9) of the BoNT/A prototoxin.

10 NIINTSILNRLRYESNHLIDLSRYASKINIGSKVNFDPIDKNQI
QLFNLESSKIEVILKNAIVNSMYENFSTSWIRIPKYFNSISLNNEYTIINCMENNS
GWKVSILNYGEIIWTLQDTQEIQRVVFVKYSQMINISDYINRWIFVTITNNRLNNSKIY
INGRLIDQKPISNLGNIHASNNIMFKLDGCRDTHRYIWIKYFNLFDKELNEKEIKDLY
DNQSNISGILKDFWGDYLYQDKPYMLNLYDPNKYVDVNVVGIRGYMYLKGPRGSVMTT
15 NIYLNSSLYRGTKFIIKKYASGNKDNIVRNDRVYINVVVKNKEYRLATNASQAGVEK
ILSALEIPDVGNLSQVVVMKSKNDQGITNKCKMNLQDNNGNDIGFIGFHQFNNIKLV
ASNWYNRQIERSSRTLGCSEFIPVDDGWGERPL

 The amino acid sequence of the BoNT/A prototoxin is
encoded by nucleotides 358 to 4245 of the neurotoxin
20 cDNA sequence, set forth herein below as SEQ ID NO: 10.

 aagcttctaa atttaaatta ttaagtataa atccaaataa acaatatgtt
caaaaacttg
25 atgaggtaat aatttctgta ttagataata tggaaaaata tatagatata
tctgaagata
atagattgca actaatagat aacaaaaata acgcaaagaa gatgataatt
agtaatgata
tatttatttc caattgttta accctatctt ataacggtaa atatatatgt
ttatctatga
30 aagatgaaaa ccataattgg atgatatgta ataatgatat gtcaaagtat
ttgtatttat
ggtcatttaa ataattaata atttaattaa ttttaaataat tataagaggt
gttaaatatg
ccatttgta ataaacaatt taattataaa gatcctgtaa atgggtgttga
35 tattgcttat
ataaaaattc caaatgcagg acaaatgcaa ccagtaaaaag cttttaaaat
tcataataaa
atatgggtta ttccagaaag agatacattt acaaatcctg aagaaggaga
tttaaatacca
40 ccaccagaag caaaacaagt tccagtttca tattatgatt caacatattt
aagtacagat
aatgaaaaag ataattattt aaagggagtt acaaaattat ttgagagaaat
ttattcaact
gatcttgtaa gaatgttggt aacatcaata gtaaggggaa taccattttg
45 gggtggaagt
acaatagata cagaattaa agttattgat actaattgta ttaatgtgat
acaaccagat
ggtagttata gatcagaaga acttaattcta gtaataatag gaccctcagc
tgatattata

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5   cagtttgaat gtaaaagctt tggacatgaa gttttgaatc ttacgcgaaa
   tggttatggc
   tctactcaat acattagatt tagcccagat tttacatttg gttttgagga
   gtcacttgaa
10  gttgatacaa atcctctttt aggtgcaggc aaatttgcta cagatccagc
   agtaacatta
   gcacatgaac ttatacatgc tggacataga ttatatggaa tagcaattaa
   tccaaatagg
   gttttttaag taaataactaa tgcctattat gaaatgagtg ggtagaagt
   aagctttgag
15  gaacttagaa catttggggg acatgatgca aagtttatag atagtttaca
   ggaaaacgaa
   tttcgtctat attattataa taagttttaa gatatagcaa gtacacttaa
   taaagctaaa
   tcaatagtag gtactactgc ttcattacag tatatgaaaa atgtttttta
20  agagaaatat
   ctctatctg aagatacatc tggaaaattt tcggtagata aattaaaatt
   tgataagtta
   tacaaaatgt taacagagat ttacacagag gataattttg ttaagttttt
   taaagtactt
25  aacagaaaaa catatttgaa ttttgataaa gccgtattta agataaatat
   agtacctaag
   gttaaattaca caatatatga tggattttaat ttaagaaata caaatttagc
   agcaaacttt
   aatgggtcaaa atacagaaat taataatatg aattttacta aactaaaaaa
30  ttttactgga
   ttgtttgaat tttataagtt gctatgtgta agagggataa taacttctaa
   aactaaatca
   ttagataaag gatacaataa ggcattaaat gatttatgta tcaaagttaa
   taattgggac
35  ttgtttttta gtccttcaga agataatttt actaatgatc taaataaagg
   agaagaaatt
   acatctgata ctaatataga agcagcagaa gaaaatatta gtttagattt
   aatacaacaa
   tattatttaa cctttaattt tgataatgaa cctgaaaata tttcaataga
40  aaatctttca
   agtgacatta taggccaatt agaacttatg cctaatatag aaagatttcc
   taatggaaaa
   aagtatgagt tagataaata tactatgttc cattatcttc gtgctcaaga
   atttgaacat
45  ggtaaattcta ggattgcttt aacaaattct gttaacgaag cattattaaa
   tcctagtcgt
   gttttatacat ttttttcttc agactatgta aagaaagtta ataaagctac
   ggaggcagct
   atgttttttag gctgggtaga acaattagta tatgatttta ccgatgaaac
50  tagcgaagta
   agtactacgg ataaaattgc ggatataact ataattattc catatatagg
   acctgcttta
   aatataggta atatgttata taaagatgat tttgtaggtg ctttaattatt
   ttcaggagct
55  gttattctgt tagaatttat accagagatt gcaataacctg tattaggtac
   ttttgcactt

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5  gtatcatata ttgcgaataa ggttctaacc gttcaaaca tagataatgc
   ttttaagtaaa
   agaaatgaaa aatgggatga ggtctataaa tatatagtaa caaatgggtt
   agcaaagggt
10 aatacacaga ttgatctaata aagaaaaaaa atgaaagaag ctttagaaaa
   tcaagcagaa
   gcaacaaagg ctataataaa ctatcagtat aatcaatata ctgaggaaga
   gaaaaataat attaatttta atattgatga ttttaagttcg aaacttaatg
   agtctataaa taaagctatg attaataata ataaattttt gaatcaatgc
   tctgtttcat atttaatgaa ttctatgatc
15 ccttatgggtg ttaaacgggtt agaagatttt gatgctagtc ttaaagatgc
   attattaaag
   tatatatatg ataataagagg aactttaatt ggtcaagtag atagattaaa
   agataaagtt
   aataatacac ttagtacaga tatacctttt cagctttcca aatacgtaga
20 taatcaaaga
   ttattatcta catttactga atatatata aatattatta atacttctat
   attgaattta
   agatatgaaa gtaatcattt aatagactta tctaggtatg catcaaaaat
   aaatatgggt
25 agtaaagtaa attttgatcc aatagataaa aatcaaattc aattatttaa
   tttagaaagt
   agtaaaattg aggtaatttt aaaaaatgct attgtatata atagtatgta
   tgaaaatttt
   agtactagct tttggataag aattcctaag tattttaaca gtataagtct
30 aaataatgaa
   tatacaataa taaattgtat ggaaaataat tcaggatgga aagtatcact
   taattatgggt
   gaaataatct ggactttaca ggatactcag gaaataaaac aaagagtagt
   ttttaaatat
35 agtcaaatga ttaatatatc agattatata aacagatgga tttttgtaac
   tatcactaat
   aatagattaa ataactctaa aatttatata aatggaagat taatagatca
   aaaaccaatt
   tcaaatttag gtaatatcca tgctagtaat aatataatgt ttaaattaga
40 tggttgtaga
   gatacacata gatatatattg gataaaatat tttaatcttt ttgataagga
   attaaatgaa
   aaagaaatca aagatttata tgataatcaa tcaaattcag gtattttaaa
   agacttttgg
45 ggtgattatt tacaatatga taaaccatac tatatgttaa atttatatga
   tccaaataaa
   tatgtcgatg taaataatgt aggtattaga ggttatatgt atcttaaagg
   gcctagaggt
   agcgtaatga ctacaaacat ttattttaaat tcaagtttgt atagggggac
50 aaaatttatt
   ataaaaaaat atgcttctgg aaataaagat aatattgtta gaaataatga
   tcgtgtatat
   attaatttag tagttaaaaa taaagaatat aggttagcta ctaatgcac
   acaggcaggc
55 gtagaaaaaa tactaagtgc attagaaata cctgatgtag gaaatctaag
   tcaagtagta

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5  gtaatgaagt caaaaaatga tcaaggaata acaaataaat gcaaaatgaa
   tttacaagat
   aataatggga atgatatagg ctttatagga tttcatcagt ttaataatat
   agctaaacta
10  gtagcaagta attggtataa tagacaaata gaaagatcta gtaggacttt
   gggttgctca
   tgggaattta ttctgtaga tgatggatgg ggagaaaggc cactgtaatt
   aatctcaaac
   tacatgagtc tgtcaagaat tttctgtaaa catccataaa aattttaaaa
   ttaatatggt
15  taagaataac tagatatgag tattgtttga actgcccctg tcaagtagac
   aggtaaaaaa
   ataaaaatta agatactatg gtctgatttc gatattctat cggagtcaga
   ccttttaact
   tttcttgtat cctttttgta ttgtaaaact ctatgtattc atcaattgca
20  agttccaatt
   agtcaaaaatt atgaaacttt ctaagataat acatttctga ttttataatt
   tcccaaaatc
   cttccatagg accattatca atacatctac caactcgaga catactttga
   gttgcgccta
25  tctcatthaag tttattcttg aaagatttac ttgtatattg aaaaccgcta
   tcaactgtgaa
   aaagtggact agcatcagga ttggaggtaa ctgctttatc aaaggtttca
   aagacaagga
   cgttgttatt tgattttcca agtacatagg aaataatgct attatcatgc
30  aaatcaagta
   tttcactcaa gtaacgcttt gtttcgtctg ttaac

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Of course, three distinct domains analogous to those described above for BoNT/A exist for all the BoNT subtypes as well as for TeNT neurotoxin; an alignment of the amino acid sequences of these holotoxins will reveal the sequence coordinates for these other neurotoxin species. Additionally, while sequence information is given above for BoNT/A, the amino acid sequences of all BoNT species and tetanus toxin TeNT are known and can easily be obtained from, for example, the NCBI Gen-Bank Web site: www.ncbi.nlm.nih.gov. The Clostridial neurotoxin nucleotide and amino acid sequences disclosed at this site are expressly incorporated by reference herein.

5 Preferably, the translocation element and the binding element of the compositions of the present invention are separated by a spacer moiety that facilitates the binding element's binding to the desired cell surface receptor. Such a spacer may comprise, for
10 example, a portion of the BoNT Hc sequence (so long as the portion does not retain the ability to bind to the BoNT or TeNT binding site of motor neurons or sensory afferent neurons), another sequence of amino acids, or a hydrocarbon moiety. The spacer moiety may also comprise
15 a proline, serine, threonine and/or cysteine-rich amino acid sequence similar or identical to a human immunoglobulin hinge region. In a preferred embodiment, the spacer region comprises the amino acid sequence of an immunoglobulin γ 1 hinge region; such a sequence has
20 the sequence (from N terminus to C terminus):

EPKSCDKTHTCPPCP (SEQ ID NO:11)

 It will be understood that none of the examples or embodiments described herein are to be construed as limiting the scope of the invention, which is defined
25 solely by the claims that conclude this specification.

Example 1:

 An agent for the treatment of acute pancreatitis is
30 constructed as follows.

 A culture of *Clostridium botulinum* is permitted to grown to confluence. The cells are then lysed and total RNA is extracted according to conventional methods and in the presence of an RNase inhibitor. The RNA
35 preparation is then passed over a oligo(dT) cellulose column, the polyadenylated messenger RNA is permitted to

5 bind, and the column is washed with 5-10 column volumes
of 20 mM Tris pH 7.6, 0.5 M NaCl, 1 mM EDTA
(ethylenediamine tetraacetic acid), 0.1% (w/v) SDS
(sodium dodecyl sulfate). Polyadenylated RNA is then
eluted with 2-3 column volumes of STE (10 mM Tris (pH
10 7.6), 1 mM EDTA, 0.05% (w/v) SDS). The pooled mRNA is
then precipitated in 2 volumes of ice cold ethanol,
pelleted in a centrifuge at 10,000 x g for 15 minutes,
then redissolved in a small volume of STE.

The BoNT/A mRNA is used as a template for DNA
15 synthesis using Moloney murine leukemia virus reverse
transcriptase (MMLV-RT), then the L chain and then H_N
chain of the neurotoxin is amplified from the cDNA by
the polymerase chain reaction (PCR) using appropriate
oligonucleotide primers whose sequences are designed
20 based on the BoNT/A neurotoxin cDNA sequence of SEQ ID
NO: 9. These procedures are performed using the
standard techniques of molecular biology as detailed in,
for example, Sambrook et al., already incorporated by
reference herein. The primer defining the beginning of
25 the coding region (5' side of the L chain fragment) is
given a StuI site. The PCR primer defining the 3' end of
the H_N-encoding domain has the following features (from
3' to 5'): a 5' region sufficiently complementary to the
3' end of the H_N-encoding domain to anneal thereto under
30 amplification conditions, a nucleotide sequence encoding
the human immunoglobulin hinge region γ_1 (SEQ ID NO:11),
a nucleotide sequence encoding the human CCK-8
octapeptide (SEQ ID NO:6), and a unique restriction
endonuclease cleavage site.

5 The PCR product (termed BoNT/AL^{HN-Y-CKK}) is purified
by agarose gel electrophoresis, and cloned into a
pBluescript II SK vector. The resulting plasmid is used
to transform competent *E. coli* cells, and a preparation
of the resulting plasmid is made. The BoNT/AL^{HN-Y-CKK}
10 fragment is excised from the pBluescript vector and
cloned into a mammalian expression vector immediately
downstream of a strong promoter. The resulting vector
is used to transfect a culture of the appropriate host
cell, which is then grown to confluence. Expression of
15 the BoNT/AL^{HN-Y-CKK} polypeptide is induced, and the cells
are lysed. The polypeptide is first purified by gel
exclusion chromatography, the fractions containing the
recombinant therapeutic agent are pooled, then the
BoNT/AL^{HN-Y-CKK} polypeptide is further purified using an
20 anti-Ig affinity column wherein the antibody is directed
to the γ_1 hinge region of a human immunoglobulin.

5 Example 2: Method of Treating a Patient Suffering from
 Acute Pancreatitis

 A therapeutically effective amount of the BoNT/A^{LHN}-
 ZCCK agent constructed and purified as set forth in
10 Example 1 is formulated in an acceptable infusion
 solution. Properties of pharmacologically acceptable
 infusion solutions, including proper electrolyte
 balance, are well known in the art. This solution is
 provided intravenously to a patient suffering from acute
15 pancreatitis on a single day over a period of one to two
 hours. Additionally, the patient is fed intravenously
 on a diet low in complex carbohydrates, complex fats and
 proteins.

 At the beginning of treatment, the patient's
20 pancreas shows signs of autodigestion, as measured by
 blood amylase levels. After the treatment regimen,
 autodigestion has ceased, and the patient's pancreas has
 stabilized.

25 Example 3: Alternative Treatment Method

 In this example, a patient suffering from acute
 pancreatitis is treated as in Example 2, with, the
 therapeutic agent given continuously over a period of
30 two weeks. After the treatment regimen, autodigestion
 has ceased, and the patient's pancreas has stabilized.

5 Example 4: Alternative Treatment Method

 In this example, a patient suffering from acute
pancreatitis is given a single pharmacologically
effective amount of the therapeutic agent of Example 1
10 by parenteral administration. Two days after the
treatment regimen, autodigestion has ceased and the
patient's pancreas has stabilized.

 It will be understood that the present invention is
15 not to be limited by the embodiments and examples
described herein, and that the invention is defined
solely by the claims that conclude this specification.

5

CLAIMS

What is claimed is:

1. A composition for the treatment of acute
10 pancreatitis in a mammal comprising,
 - a. a first element comprising a binding
element able to specifically bind a pancreatic
cell surface marker under physiological
conditions,
 - 15 b. a second element comprising a
translocation element able to facilitate the
transfer of a polypeptide across a vesicular
membrane, and
 - c. a third element comprising a therapeutic
20 element able, when present in the cytoplasm of
a pancreatic cell, to inhibit enzymatic
secretion by said pancreatic cell.
2. The composition of claim 1 wherein said pancreatic
25 cell is an acinar cell and said cell surface marker
is a CCK receptor.
3. The composition of claim 1 wherein said therapeutic
30 element will cleave a SNARE protein and cleavage of
said SNARE protein inhibits said secretion.
4. The composition of claim 3 wherein said SNARE
protein is selected from the group consisting of
syntaxin, SNAP-25 and VAMP.

35

- 5 5. The composition of claim 2 wherein said therapeutic
 element will cleave a SNARE protein, wherein
 cleavage of said SNARE protein inhibits said
 secretion.
- 10 6. The composition of claim 5 wherein said SNARE
 protein is selected from the group consisting of
 syntaxin, SNAP-25 and VAMP.
7. The composition of claim 5 wherein said CCK
15 receptor is the human CCK A receptor.
8. The composition of claim 7 wherein the binding
 element of said thereapeutic polypeptide comprises
 a human CCK A amino acid sequence modified by the
20 presence of a C-terminal amidated phenylalanine and
 a sulfated tyrosine at the position 7 residues from
 the carboxyl terminus.
9. The composition of claim 8 wherein said CCK A amino
25 acid sequence comprises SEQ ID NO: 6.
10. The composition of claim 9 wherein said CCK A amino
 acid sequence comprises SEQ ID NO: 5.
- 30 11. The composition of claim 9 wherein said CCK A amino
 acid sequence comprises SEQ ID NO: 4.
12. The composition of claim 9 wherein said CCK A amino
 acid sequence comprises SEQ ID NO: 3.

35

- 5 13. The composition of claim 9 wherein said CCK A amino
 acid sequence comprises SEQ ID NO: 2.
14. A method for making a therapeutic polypeptide
 having a binding element selective for a CCK
10 receptor comprising:
- a) expressing within a host cell a recombinant
 chimeric polypeptide comprising an extein
 comprising a therapeutic element and a
 translocational element, and an intein located to
15 the carboxyl terminal side of said extein having at
 its amino terminus an first amino acid selected
 from the group consisting of cysteine, serine or
 threonine,
- b) contacting said extein with
- 20 c) a synthetic peptide comprising a CCK amino
 acid sequence containing modifications comprising
 the presence of an amidated phenylalanine at a
 natural C-terminus of said sequence, and further
 containing at an N-terminus a second amino acid
25 selected from the group consisting of cysteine,
 serine or threonine,
- ii) a nucleophilic reagent able to
 cause cleavage of said intein from the C-
 terminus of said extein and the
30 subsequent formation of a peptide bond
 between said extein C-terminus and the N-
 terminus of said synthetic peptide
 through the formation of an activated
 ester or thioester intermediate.
- 35

- 5 15. The method of claim 14 wherein said first and second amino acids are cysteine.
16. The method of claim 15 wherein said nucleophilic reagent is selected from the group consisting of
10 phenol or thiphenol.
17. The method of claim 14 wherein said synthetic polypeptide further comprises a sulfated tyrosine at the position 7 amino acids from a natural C
15 terminus of said sequence, and said therapeutic polypeptide preferentially binds a CCK-A receptor.
18. The method of claim 17 wherein said first and second amino acids are cysteine.
- 20 19. The method of claim 18 wherein said nucleophilic reagent is selected from the group consisting of phenol or thiphenol.

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Sachs, George

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(54) Title: **METHODS AND COMPOSITIONS FOR THE TREATMENT OF PANCREATITIS**

(57) Abstract: Methods and compositions for the treatment of acute pancreatitis in a mammal. Particular compositions comprise a binding element, a translocation element, and a therapeutic element able to prevent accumulation of digestive enzymes within the pancreas.



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INTERNATIONAL SEARCH REPORT

Int. Application No

PCT/US 00/09142

A. CLASSIFICATION OF SUBJECT MATTER
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B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

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Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

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C. DOCUMENTS CONSIDERED TO BE RELEVANT

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Y		1-18
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☒ Further documents are listed in the continuation of box C.

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INTERNATIONAL SEARCH REPORT

International Application No

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

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